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The ability of purified glycosphingolipids to enhance liposome stimulated *Entamoeba histolytica* actin polymerization was assessed as a means to define the specificity of mammalian cell membrane lipid glycan recognition by this parasite. Synthetic liposomes containing a variety of individual glycosphingolipids bearing neutral, straight chain oligomeric glycans with galactose or N-acetylgalactosamine termini stimulated rapid (90 sec) polymerization of amoeba actin. Glycans with terminal N-acetylglucosamine residues were not, or only weakly, stimulatory. Glycans with glucose, N-acetylglucosamine, galactose and N-acetylgalactosamine as the penultimate residue were recognized. Attachment of N-acetylneuraminate to the terminal residue of a stimulatory glycosphingolipid eliminated activity; attachment of fucose to the penultimate sugar reduced activity. Glycans with a terminal β1-4 or 1-3 glycosidic bond were most effective; glycans with terminal α1-4 or 1-3 glycosides were less effective. The activity of glycans with both β and α-linked terminal glycosides was inhibited by lactose, suggesting recognition of both configurations by a single amoeba protein. The ability of liposomes to stimulate actin polymerization reflected the extent of liposome phagocytosis.

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INTRODUCTION

Scope of report

Accomplishments during the first half of this contract include the completion of Research Objectives 1 and 2. These are to: 1) Identify glycosphingolipid molecules of the membrane of mammalian target cells that stimulate phagocytic attack by *E. histolytica*; 2) Construct chemically defined liposomes that are selectively phagocytized by *E. histolytica*. Work has also been initiated on Objective 3: to determine the ability of amoeba specific liposomes to deliver drugs to and selectively destroy *E. histolytica* trophozoites in cultures of mammalian cells *in vitro*. Results of work on Objectives 1 and 2 have recently been published (Bailey *et al.*, 1990. Specificity of glycosphingolipid recognition by *Entamoeba histolytica* trophozoites. Infection and Immunity, 58:43-47) and are the subject of this report.

Report Introduction

Interaction of *Entamoeba histolytica* with mammalian cells is believed to be initiated by binding of amoeba membrane proteins to target cell surface carbohydrates (7, 13). The primary evidence for this is the inhibition by sugars of amoeba attachment and destruction of model target cells *in vitro*. The most effective monosaccharides are galactose (Gal) and N-acetylgalactosamine (GalNAc) (13). The most effective disaccharides are lactose (Lac) (3) and N-acetyllactosamine (LacNAc) (9). N-Acetylglucosamine (GlcNAc) and its oligomers, and melibiose (Galα1-6Glc), have also been reported to inhibit by some authors (3, 8, 11, 15) and not to by others (9).

Chinese hamster ovary (CHO) cell mutants with defined alterations of surface glycan sequences have also been used to explore *E. histolytica* carbohydrate recognition specificity. Li et al. (9) employed a panel of lectin resistant mutants with altered Asn-linked carbohydrate chains to study target cell adherence to *E. histolytica*. Wild-type CHO cells contain the terminal sequence, NeuAcα2-3Galβ1-4GlcNAcβ1-3Man-. Mutants lacking the terminal NeuAc (increased Galβ1-4 termini) adhered more effectively to *E. histolytica* trophozoites at 4^{o}C than did wild-type cells or mutants lacking the terminal NeuAc-Gal (increased GlcNAc termini) or more of the terminal sequence (9). Ravdin et al. (14) assessed adherence and cytolysis of a panel of CHO cell mutants with alterations of both Asn-linked and Ser/Thr-linked glycans. They

found that adherence and cytolysis were greatest with mutants bearing increased Galβ1-4 termini. On the basis of these findings and sugar inhibition studies, these groups concluded that N-acetyllactosamine units (Galβ1-4GlcNAc) were recognized most specifically by *E. histolytica*, a possibility that had been suggested earlier by Cano-Mancera and Lopez-Revilla (3).

To date, studies have focused on *E. histolytica* recognition of protein linked glycoconjugates. We demonstrated that liposomes prepared from human red blood cell (RBC) membrane lipids stimulate the same rapid, contact dependent polymerization of *E. histolytica* actin and phagocytosis that is triggered by contact with whole target cells (1, 2). The response to membrane liposomes was inhibited by the same sugars that block amoeba-whole cell interactions, suggesting that it was mediated by recognition of membrane glycosphingolipid glycans expressed on the surface of the vesicles (2). Here we describe the ability of synthetic liposomes formulated with a panel of individual membrane glycosphingolipids of known glycan sequence to stimulate amoeba actin polymerization. The intensity of the parasite's response was strongly affected by the structure of the glycosphingolipid glycan presented. The results demonstrate that *E. histolytica* interacts with mammalian cell membrane glycosphingolipids in liposomes and define glycosphingolipid glycan recognition specificity.

BODY

Experimental Methods

The following abbreviations are used: PBSS, 15 mM potassium phosphate-150 mM NaCl-5 mM $MgCl_2$-2 mM $CaCl_2$, pH 6.3; Gal, galactose; Glc, glucose; Man, mannose; Lac, lactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; NeuAc, N-acetylneuraminate; LacNAc, N-acetyllactosamine.

Commercial lipids and sugars were obtained from Sigma Chemical Co., St. Louis, MO. Lipids were identified and checked for purity by TLC as described previously (2). Rhodamine-phalloidin was purchased from Molecular Probes, Eugene, OR.

Amoebae. *E. histolytica*, strain HM1-IMSS, was cultured axenically in TYI-S-33 medium (5) as described (1).

Glycosphingolipids. Glucosylceramide, galactosylceramide, lactosylceramide trihexosylceramide (CTH), (GlcNAc)trihexosylceramide ((GlcNAc)CTH), globoside, paragloboside (PG), Forssman, norhexaosylceramide (NHC), GM_3, and sialylparagloboside (SPG) were all prepared from human erythrocyte stroma as follows. Erythrocytes were lysed in hypotonic 0.1% acetic acid on ice and centrifuged. The washed pellet was homogenized in isopropanol:hexane:water (I:H:W) 55:25:20 v/v, and filtered. The organic extract filtrate was evaporated to dryness and brought up in a large volume of chloroform:methanol (C:M), 2:1, whereupon 1/6 volume of deionized water was added and the suspension inverted and mixed well. After the phases separated, the upper phase was drawn off, evaporated to dryness, transferred in water to Spectrapore dialysis tubing (MW cutoff 2500) and dialyzed against water. The lower phase was also evaporated to dryness and brought up in a small volume of C:M. After dialysis of the upper phase for two days, this fraction was lyophilized and brought up in chloroform:methanol:water (C:M:W), 30:60:8.

Lower phase glycolipid preparation/HPLC chromatography: The lower phase was applied to an Iatrobeads column (porous silica gel, 10 μm diameter, Iatron, Tokyo), on a Varian HPLC and eluted with a gradient of I:H:W (55:40:5 to 55:25:20) at 2ml/min over 4 hr. In this manner, CMH, CDH, CTH and globoside were isolated in pure quantities. An impure Forssman fraction was subsequently purified by additional HPLC runs.

Upper phase. A DEAE Sephadex column (A-25, Sigma) was prepared after equilibrating the Sephadex in C:M:0.8 M sodium acetate, 30:60:8, overnight and washing in C:M:W, 30:60:8. The

upper phase, dissolved in C:M:W, 30:60:8, was applied to the column and washed extensively with this solvent, then with MeOH. The monosialyl gangliosides were eluted with 0.05 M ammonium acetate in MeOH. This fraction was evaporated to dryness, transferred to a dialysis bag in water and dialysed 3 days. The dialyzed fraction was lyophilized and brought up in a small volume of C:M, 2:1.

HPLC of the monosialyl fraction. The fraction was chromatographed on an Iatrobeads column as above, and GM_3, SPG, and sialylnorhexaosylceramide (SNHC) were isolated in pure quantities. The SPG and SNHC were cleaved to PG and NHC, respectively by heating in aqueous 1% acetic acid at 100°C for 1 hr. (GlcNAc)CTH and agalactosyl-NHC were prepared by enzymatic cleavage of PG and NHC using jackbean β-galactosidase (Sigma) in 0.2 M citrate, pH 4.0, overnight at 37°C.

Le^x pentasaccharide and dimeric Le^x were prepared from human colonic adenocarcinoma upper neutral fraction by HPLC in a manner similar to that above. Rabbit afucosyl B antigen was prepared from rabbit erythrocytes in the same way.

Preparation of Liposomes. RBC membrane liposomes were prepared by sonication as previously described (2). The total lipid concentration of RBC membrane liposome suspensions was the same as that of synthetic liposomes based on total phosphorous content (6). Synthetic liposome suspensions contained 2.5 mM cholesterol, 2 mM sphingomyelin, 1.3 mM phosphatidylethanolamine, 1.2 mM dipalmityl phosphatidylcholine, 0.6 mM phosphatidylserine and 0.33 mM glycosphingolipid. Lipids, first dissolved and mixed in C:M:W (50:25:1), were dried at 45°C and sonicated in PBSS as described previously (2). Liposome suspensions were used within one week of preparation.

Assay of liposome stimulated amoeba actin polymerization. A simplification of the method described earlier (2) was used. Amoebae were washed and resuspended in PBSS at a concentration of 10^6 cells/ml. One hundred µl was placed in wells of a conical bottom 96-well plate, incubated at 25°C for 5 min, then challenged with 10 µl/well of liposome suspension. The amoebae were resuspended at 30 sec intervals during a 90 sec challenge, then fixed with an equal volume of 0.1% glutaraldehyde-7% formaldehyde in PBS. The cells were washed 5 times with PBS-0.1% Triton X-100 and stained with 0.17 µM rhodamine-phalloidin (1). Stimulation of *E. histolytica* actin polymerization was determined by counting the fraction of stimulated cells as previously described (2). RBC membrane liposomes and glycosphingolipid-free synthetic

liposomes were included in each assay for comparisons. Relative stimulation was the fraction of cells stimulated by the test liposomes divided by the fraction stimulated by RBC membrane liposomes in the same assay. The values shown in the Figures are means ± SEM for the number of assays listed in Table 1. Statistical significance was determined using the student's t-test.

Liposome phagocytosis. Liposome phagocytosis was measured with carboxyfluorescein loaded liposomes as previously described (2).

Results

Stimulation of *E. histolytica* actin polymerization by liposomes. The glycosphingolipids assessed in this study are listed in Table 1 with a key to the letter codes used in the Figures. The number of actin stimulation assays conducted with each glycosphingolipid is indicated in parentheses in Table 1.

The ability of Gal and GalNAc terminal straight chain glycosphingolipids to enhance liposome activated *E. histolytica* actin polymerization is shown in Fig. 1. Stimulation by synthetic liposomes lacking added glycosphingolipid (pl) averaged 19% of the stimulation by liposomes prepared from the total lipid extract of RBC membranes (rbc). Synthetic liposomes constructed with either of the monohexosylceramides, glucosylceramide (A) or galactosylceramide (B), were without stimulatory activity. In fact, glucosylceramide depressed stimulatory activity below that of the glycolipid-free liposomes ($P<0.01$).

All Gal or GalNAc terminal glycosphingolipids bearing two or more sugars enhanced the ability of the synthetic liposomes to stimulate *E. histolytica* actin polymerization compared to the glycolipid-free liposomes ($P < 0.001$). Glycans with a β1-4 or 1-3 terminal glycosidic bond (L, F, C, G) were more effective than glycans with a terminal α1-4 or 1-3 linkage (H, D, I). Liposomes constructed with trihexaosylceramide (D, Galα1-4 terminus) and rabbit afucosyl B antigen (H, Galα1-3 terminus) were only about 50% as effective as RBC membrane liposomes ($P < 0.05$).

The kinetics of synthetic liposome stimulated cytoskeleton activation were determined using paragloboside liposomes. As with RBC membrane liposomes (2), the maximum stimulation was reached between 1 and 2 min following challenge (data not shown). No stimulation of actin polymerization occurred with paragloboside bearing liposomes if phosphatidylserine was omitted

TABLE 1. Glycosphingolipids tested.

Fig. label	Name and formula (number of times assayed)
pl.	Glycosphingolipid-free liposomes (56)
rbc.	RBC membrane liposomes (56)
A.	Glucosylceramide (6) Glcβ1-ceramide
B.	Galactosylceramide (6) Galβ1-ceramide
C.	Lactosylceramide (19) Galβ1-4Glcβ1-ceramide
D.	Trihexaosylceramide (19) Galα1-4Galβ1-4Glcβ1-ceramide
E.	(GlcNAc)trihexaosylceramide (7) GlcNAcβ1-3Galβ1-4Glcβ1-ceramide
F.	Globoside (19) GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-ceramide
G.	Paragloboside (40) Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide
H.	Rabbit afucosyl B antigen (6) Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide
I.	Forssman (12) GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-ceramide
J.	Agalactosyl-norhexaosylceramide (12) GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide
K.	Le^x pentasaccharide (4) Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-ceramide
L.	Norhexaosylceramide (18) Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide
M.	Dimeric Le^x (9) Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-ceramide
N.	GM_3 (8) NeuAcα2-3Galβ1-4Glcβ1-ceramide
O.	Sialylparagloboside (8) NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide

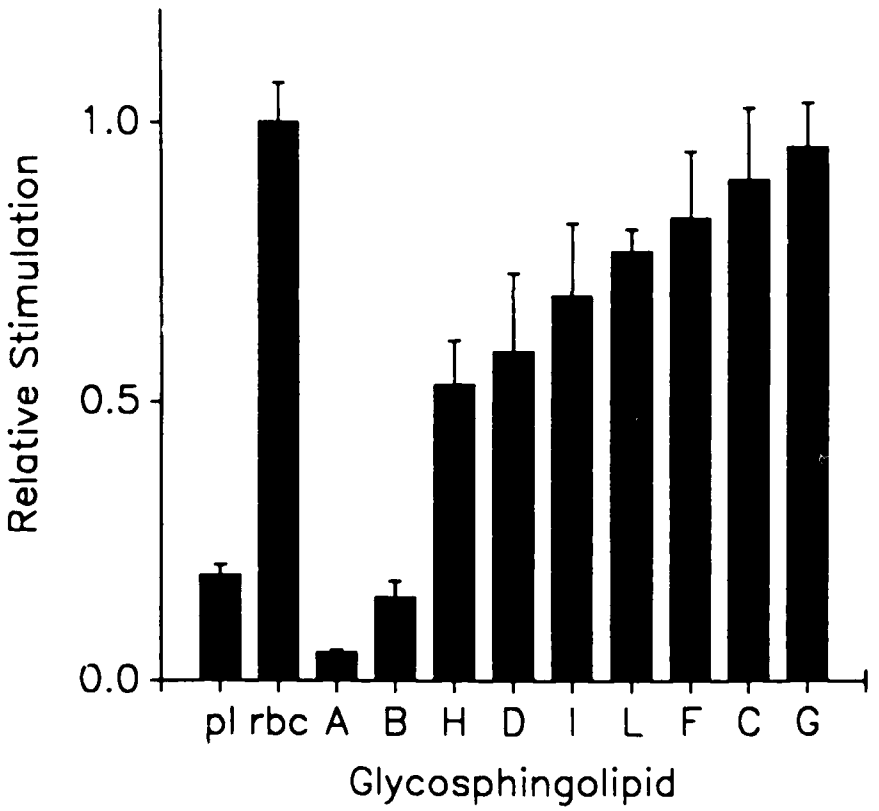

Fig. 1. Enhancement of liposome stimulated *E. histolytica* actin polymerization by Gal and GalNAc terminal straight chain glycosphingolipids. The key to lettered glycosphingolipids is given in Table 1.

from the liposome formulation, verifying the requirement for a negatively charged phospholipid for this response (2).

The effects of modification of glycosphingolipid glycan structure on liposome stimulatory activity are shown in Fig. 2. NeuAc attached to the terminal Gal of lactosylceramide or paragloboside (GM_3 [N] and sialylparagloboside [O], respectively) essentially eliminated the enhancing activity of the these glycosphingolipids. Fucose, attached to the penultimate sugar (GlcNAc) of paragloboside (Le^x pentasaccharide, K) or both GlcNAc residues of norhexaosylceramide (dimeric Le^x, M), was less nindering, but diminished the activity of the corresponding straight chain glycosphingolipids 56% and 38%, respectively ($P < 0.05$).

Finally, removal of the terminal Gal from paragloboside and from norhexaosylceramide, which produced (GlcNAc)trihexaosylceramide (E) and agalactosyl-norhexaosylceramide (J), respectively, eliminated the stimulatory activity of the former and reduced that of the latter 61% ($P < 0.001$). However, agalactosyl norhexaosylceramide still enhanced liposome stimulatory activity compared to the glycolipid-free controls ($P < 0.01$).

Sugar inhibition of glycosphingolipid stimulated actin polymerization. Lactose (Galβ1-4Glc), known to exhibit structurally specific inhibition of *E. histolytica* interaction with whole target cells, and glucose, ineffective as an inhibitor, were tested for their ability to inhibit amoeba actin polymerization stimulated by liposomes prepared with glycosphingolipids of opposite terminal glycoside configuration -- paragloboside (Galβ1-4GlcNAc) and Forssman (GalNAcα1-4Gal). The results are shown in Fig. 3. Lactose, but not the control sugar, inhibited the activity of RBC membrane and paragloboside (G) liposomes similarly, near 40%, and blocked the weaker stimulation by Forssman (I) liposomes 70% ($P < 0.01$).

Correlation of stimulation of actin polymerization with phagocytosis. The relative stimulation of actin polymerization reflected the relative extent of vesicle phagocytosis for pl, rbc and paragloboside (G) liposomes (Fig. 4). It was expected that a similar correlation would have been observed for the other synthetic liposome types, but this was not tested.

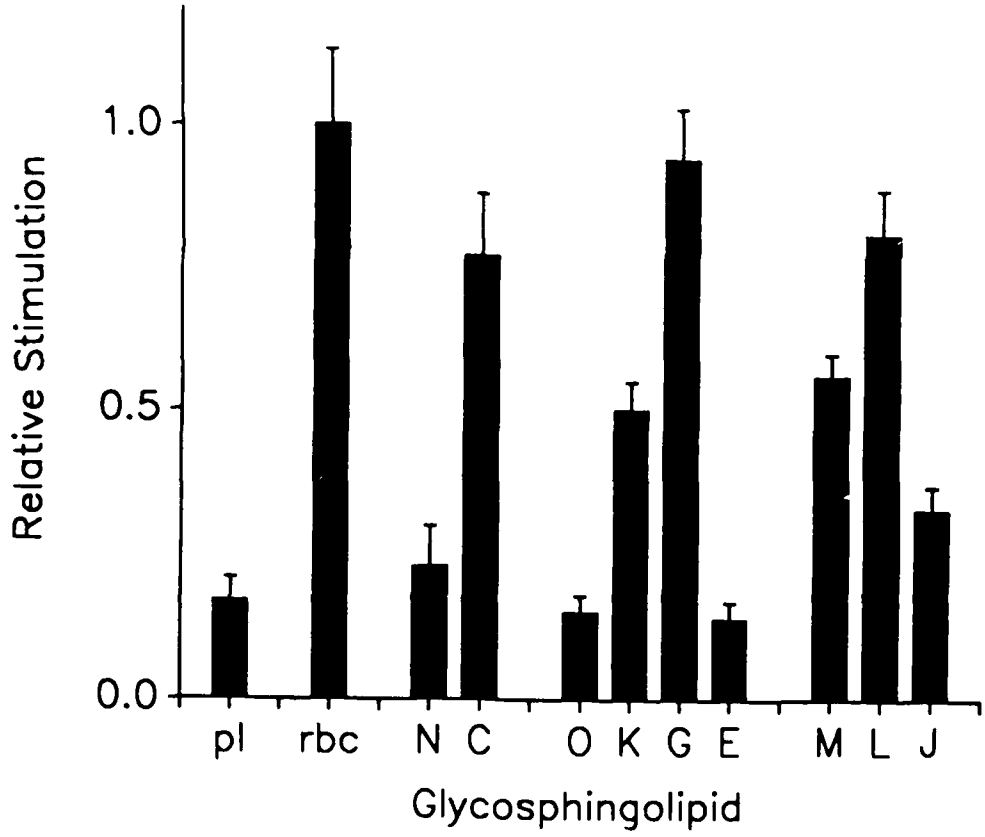

Fig. 2. The effects of structural modifications on the ability of glycosphingolipids to enhance liposome stimulated *E. histolytica* actin polymerization. The key to lettered glycosphingolipids is given in Table 1.

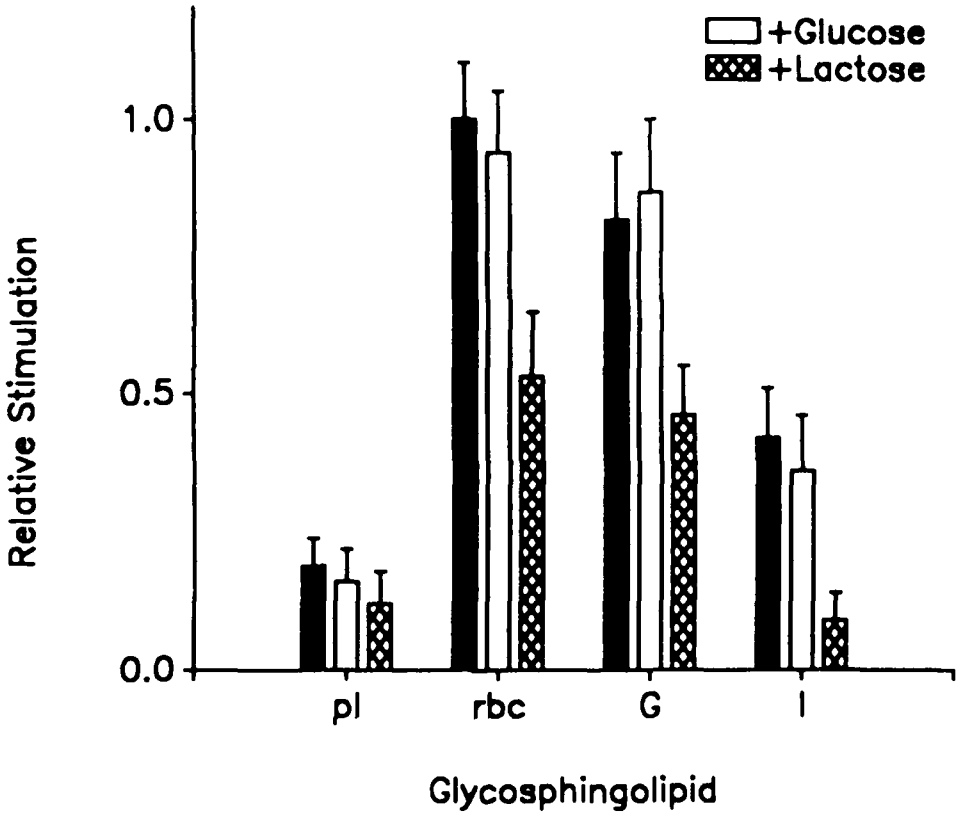

Fig. 3. The effects of glucose (50 mM) and lactose (50 mM) on the ability of RBC membrane (rbc), paragloboside (G) and Forssman (I) bearing liposomes to stimulate *E. histolytica* actin polymerization.

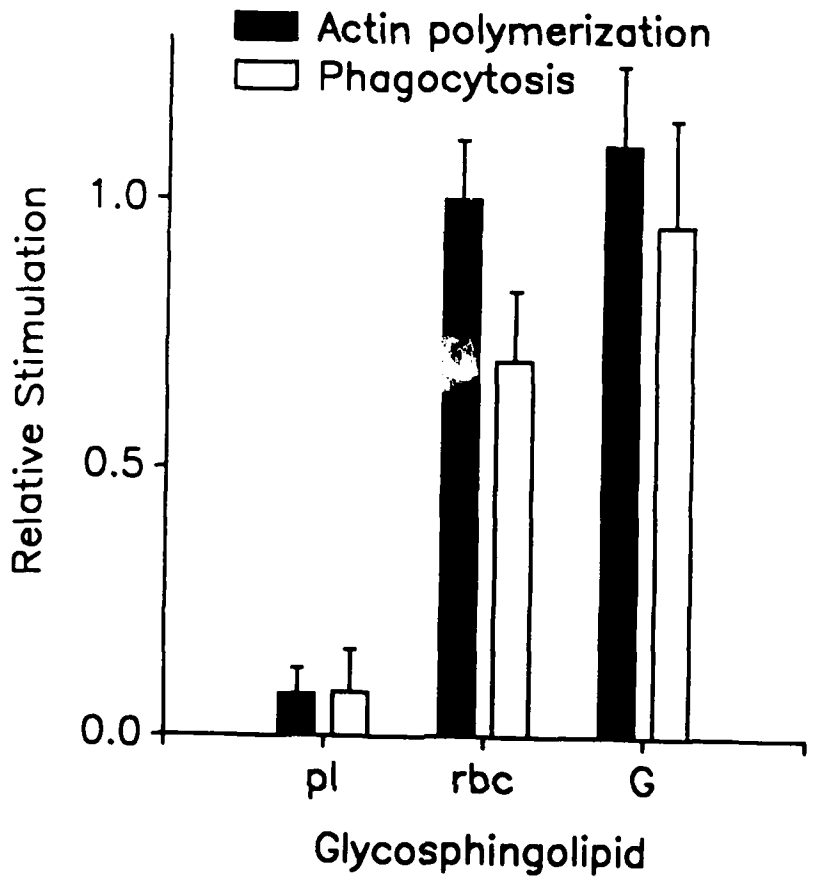

Fig. 4. Correlation of liposome stimulated *E. histolytica* actin polymerization with liposome phagocytosis. G, paragloboside.

DISCUSSION AND CONCLUSIONS

We showed previously that liposomes prepared from RBC membrane lipids mimicked whole RBCs in their ability to elicit rapid contact dependent actin polymerization and phagocytosis by *E. histolytica* (2). In the present study we have demonstrated that the same cellular responses are triggered by a variety of synthetic liposomes containing glycosphingolipid glycans. The similarities in the responses to synthetic and cell membrane liposomes included the kinetics and extent of cytoskeleton activation, a correlation of the cytoskeleton response with target phagocytosis and inhibition by a disaccharide (lactose) that blocks interaction of the parasite with cell membrane liposomes and whole cells. Based on this evidence, we have concluded that the relative degree to which synthetic liposomes stimulated amoeba actin polymerization reflected the relative specificity of the parasite for interaction with the lipid associated glycoconjugates expressed on these vesicles.

E. histolytica recognized a variety of glycosphingolipid glycans with Gal or GalNAc terminal residues. A β1-4 or 1-3 terminal glycosidic bond was favored. Glycosphingolipids with an α1-4 or 1-3 terminal linkage possessed some, but weaker, stimulatory activity. The structure of the penultimate sugar of the glycoconjugate was not critical for recognition, since this position was occupied by GlcNAc, Glc, GalNAc and Gal in different stimulatory glycans. The failure of galactosylceramide to enhance liposome stimulated actin polymerization implies that a disaccharide glycan is minimally essential. However, as suggested earlier (2), the failure of this monohexosylceramide to stimulate a response may have been due to hindrance of carbohydrate binding interactions close to the lipid bilayer. The apparent inhibition of the basal activity of glycolipid-free liposomes by glucosylceramide is intriguing, but unexplained.

Terminal GlcNAc residues appeared not to be recognized. Removal of the terminal Gal from paragloboside eliminated the activity of this, the most stimulatory of the glycosphingolipids tested. Loss of the terminal Gal from norhexaosylceramide reduced its activity by 61%. The residual activity of agalactosyl-norhexaosylceramide may have reflected recognition of the internal Galβ1-4 residue of that glycan.

NeuAc attached to the Gal terminus of an otherwise stimulatory glycosphingolipid or fucose attached to the next proximal sugar significantly reduced stimulatory activity. With fucose, this was probably due primarily to steric hindrance of

binding to the terminal sugar; in the case of the negatively charged NeuAc, repulsive charge effects may be involved, since the liposomes also carry a net negative charge.

Our results are generally consistent with those obtained in the studies of *E. histolytica* adherence to CHO cell surface glycosylation mutants (9, 10, 14). Interaction was always greatest with Galβ1-4 terminal glycans unencumbered by attached NeuAc or fucose residues. Interaction with GlcNAc terminal glycans was low. In addition we have detected recognition of terminal Gal(GalNAc)β1-3 and Gal(GalNAc)α1-4 or 1-3 glycans.

Li *et al.* (9) proposed that terminal Galβ1-4GlcNAc units represented the principal carbohydrate structure recognized by *E. histolytica*. This conclusion was based on the observation of maximal adherence of amoebae to mutants with increased Galβ1-4GlcNAc termini and, of a number of sugars tested, the strongest inhibition of adherence (9) and cytolysis (10) by LacNAc. Ravdin and co-workers reached the same conclusion (14). Our results support this, but also have demonstrated by analysis of individual glycans that other terminal sequences and configurations are recognized, at least when presented as glycosphingolipids in the liposome model.

While other interpretations are possible, the ability of lactose to inhibit amoeba interaction with both paragloboside and Forssman glycosphingolipid bearing liposomes implies that a single binding protein was responsible for recognition of all the glycosphingolipids we tested. A logical candidate is the Gal/GalNAc binding protein which has been studied extensively and isolated by Petri and co-workers (12). The greater percentage inhibition and weaker cytoskeleton stimulating activity of glycans with an α-linked terminal glycoside imply weaker affinity of the binding protein for this configuration than for β-linked terminal residues. A further implication of our results, in general, is that binding occurs primarily to the terminal sugar(s) of lipid glycans.

Previous investigations have focused on recognition of mammalian cell glycoproteins by *E. histolytica*. Recognition specific binding and ingestion of galactose terminal intestinal mucins have been demonstrated (), and we have shown enhanced attachment and phagocytosis by *E. histolytica* of latex beads conjugated with galactose terminal glycoproteins (Bailey, G. B., *et al.*, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989). The present study demonstrates that glycosphingolipids prepared from target cell membranes are recognized by the parasite. Because of common

steps in biosynthesis, some mutations cause the same alteration of both protein and lipid glycoconjugates in CHO cells (16). When such mutants have been used in studies of *E. histolytica* carbohydrate recognition specificity, the results may have reflected alterations in glycosphingolipid as well as glycoprotein glycan structure.

Because glycolipid-free liposomes stimulate some amoeba actin polymerization, and because stimulatory glycosphingolipid bearing liposomes lacking a negatively charged phospholipid are inactive, it is clear that binding of glycosphingolipid glycans *per se* to amoeba proteins does not trigger the parasite cytoskeleton response. Presumably, glycan binding facilitates interactions with other molecules of the vesicle lipid bilayer. It is important now to determine whether *E. histolytica* distinguishes glycoprotein from glycosphingolipid glycans on the surface of mammalian cells and, if so, the relevance this has to the mechanism of target cell attack by the parasite.

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